Spores IV

Activation of *Bacillus megaterium* Spore Germination

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Dormant spores of *Bacillus megaterium* QM B1551 could be activated for germination on glucose by heating in aqueous suspension (but not if heated dry), by treating with aqueous ethyl alcohol at 30 C, or by exposure to water vapor at room temperatures. It is postulated that activation involves hydration of a critical (enzymatic?) spore site, and that heating and ethyl alcohol modify the molecular or physical properties of liquid water so as to resemble water vapor in ability to reach or react with this site, or to do both. The germination characteristics of spores converted to various ionic forms by base-exchange techniques were also examined. The conversion of native spores to the H form by acid treatment had a dual effect: (i) decreased germination on glucose, and increased germination on L-alanine; and (ii) sensitization of germination to heat inactivation. Loading of H-form spores with Ca^{++} (or other cations) reversed these changes. The germination pattern in response to heating represented a balance between activation and inactivation, which was strongly influenced by exchangeable spore cations.

Activation, a reversible process, results in a spore which retains its typical heat resistance, refractility, nonstainability, and dipicolinic acid (DPA) content, but which is no longer dormant. Activation increases the rate and extent of germination, activates enzymes which are dormant in resting spores, and changes the requirements for the induction of germination (13). Activation requirements depend upon the sporulation medium, the chemical composition of the spore, its storage history, and the nature of the germination agent.

Curran and Evans (3) first clearly recognized that bacterial spores could be activated by the application of heat. Later workers found that spores were also activated by reducing agents and by low *p*H (12). Spores of *Bacillus stearothermophilus*, activated for increased colony count by acid treatment, could be reverted to dormancy by exposure to Ca^{++} at *p*H 9 (16). On the other hand, spores of *B. megaterium*, activated for spontaneous germination by exposure to Ca-DPA, were deactivated by acid treatment (14). The presence of various cations affected the rate at which dor-

mant *B. popilliae* spores were heat-activated (22). Reversible changes in *B. megaterium* germinability depended upon calcium and other cations (19, 20).

In this paper, we first summarize our previous work showing that *B. megaterium* QM B1551 spores were activated by heat, by aqueous ethyl alcohol, and by water vapor (11). We have postulated that heating and ethyl alcohol change the structure of liquid water so that it is more like water vapor and can more readily penetrate to and hydrate a critical (enzymatic?) spore site, leading to activation. Second, we report previously unpublished studies on various ionic forms (1) of *B. megaterium* QM B1551 spores—their heat sensitivity, their heat activation, and their germination in glucose, KI, or L-alanine.

MATERIALS AND METHODS

Spore preparation. Spores of *B. megaterium* QM B1551, prepared on the complex medium (omitting agar) of Arret and Kirshbaum (2) were harvested, washed, and lyophilized. The spores were stored, at 4 C, in a desiccator over either $CaSO_4$ or silica gel. Spores from several harvests were pooled. These spores contained (dry weight basis) 2.6% calcium, 10% DPA, and ca. 5% water (removable in 24 hr either by drying at 100 C or by evacuation).

Activation by heat, aqueous ethyl alcohol, and water vapor. The spore activation methods have been described (11). Briefly, for heat activation, 10-ml amounts of spores in aqueous suspension (2 mg of spores/ml) were heated for 10 min in water baths at the indicated temperatures. Activation was stopped by chilling in an ice bath. Similar spore suspensions (10 ml) were activated by exposure to varying concentrations of aqueous ethyl alcohol at 30 C for 5 min. Activation was arrested by discarding the alcoholic supernatant fraction after centrifugation at 4 C, washing the spore pellet with 40 ml of water, and suspending it in 10 ml of water. For activation by water vapor, lyophilized spores were coated onto the surface of no. 13 Ballotini beads, and were exposed for 120 min at 30 C in small petri dishes in desiccators previously equilibrated to the desired relative humidities with various H_2SO_4-water controlling solutions. Activation was stopped by the addition of 10 ml of liquid water.

The extent of activation was determined by measuring the increase in the rate and extent of germination (over that of the untreated spores) at 30 C, in glucose (25 mM), KI (5 mM) or L-alanine (100 mM), buffered at *p*H 7.0 with 50 mM potassium phosphate. Germination was followed kinetically by decrease in optical density (OD) of spore suspensions (0.35 to 0.4 mg of spores/ml) at 560 nm (Klett-Summerson colorimeter). A 25% loss in OD approximated 50% germination (stainability); loss of 55 to 60% of the original OD corresponded to 98 to 100% germination. The germination rate was calculated as the highest percentage decrease in OD per minute after the addition of substrate.

Deactivation and reactivation. Activated spores were centrifuged and washed with water before deactivation. Spore pellets were lyophilized for 4 hr, and then exposed in evacuated desiccators over P_2O_5 for 2 hr at 30 C, and for an additional 40 hr at 66 C. Reactivation methods are indicated with the appropriate results.

Preparation of spores of different ionic form. Aqueous suspensions of pooled spores (10 mg of spores/ml) were converted to the hydrogen form ("H-spores") by titration to *p*H 6.0, 4.0, or 2.5 with 0.01 N HNO_3 (1). They were continually stirred at 25 C and maintained at these *p*H levels for 3 hr by frequent additions of acid. The suspensions were then centrifuged, washed three times, and lyophilized. These "stripped" spores are referred to as H-6, H-4, or H-2.5 spores (Table 1). Microscopic appearance and negligible release of DPA (less than 2% of the total) indicated that these spores remained stable and ungerminated. Spores, not treated with acid, but similarly washed and lyophilized, are designated as native spores ("N-spores").

Spores "loaded" with Ca^{++}, K^{+}, Na^{+}, Sr^{++}, or Ba^{++} were prepared by titrating aqueous suspensions of H- or N- spores to *p*H 9.0 with the hydroxides of these metals and maintaining them at this *p*H for 3 hr at 25 C with continual stirring, before washing and lyophilizing (Table 1). The amount of acid or alkali consumed in preparation of these spores is indicated in Table 1. The *p*H of aqueous spore suspensions (1 mg of spores/ml) was at 7.0 $\pm$ 0.5, regardless of ionic form.

Heat activation of spores of different ionic form. To decrease the heating lag, a small volume (0.3 ml) of heavy spore suspension (30 mg of spores/ml) was added to 8.7 ml of water, pre-equilibriated to the desired temperature. After 10 min of heating, the suspension (1 mg of spores/ml) was chilled in an ice bath.

Heat resistance. The heat resistance of spores was determined by conventional plating techniques. Aqueous suspensions (0.05 mg of spores/ml) were heated at 84 C for 5 min, and the percentage survival was calculated from colony counts after 24 hr of incubation at 30 C on Nutrient Agar, supplemented with 0.1% yeast extract.

Table 1. *Preparation of ionic forms of spores of B. megaterium QM B1551*[a]

Ionic form	*p*H adjusted with	Acid or alkali consumed (meq/g of spores)
H-6	HNO_3	0.188
H-4	HNO_3	0.658
H-2.5	HNO_3	2.190
N-Ca	$Ca(OH)_2$	0.184
H-4-Ca	$Ca(OH)_2$	0.720
H-2.5-Ca	$Ca(OH)_2$	1.020
N-K	KOH	0.168
H-4-K	KOH	0.600
N-Na	NaOH	0.140
H-4-Na	NaOH	0.550
H-4-Sr	$Sr(OH)_2$	0.760
H-4-Ba	$Ba(OH)_2$	0.770

[a]Pooled spores (10 mg of spores/ml) were converted to the H-form by titration at 25 C to the indicated *p*H with 0.01 N HNO_3 and maintenance at that *p*H for 3 hr; N-spores (native spores) were prepared by similar treatment of pooled spores with water; cation-loaded spores (e.g., Ca-spores) were prepared similarly by titration to *p*H 9 of N- or H-4 spores with hydroxides of the appropriate metal. All spore preparations were washed three times and lyophilized.

RESULTS AND DISCUSSION

Activation by heat, water vapor, and aqueous ethyl alcohol. We have reported (11) that untreated *B. megaterium* spores were essentially dormant for glucose-induced germination (Fig. 1). After 120 min of incubation in 25 mM glucose, only about 8% of them had germinated (4% OD loss). However, spores which had been heated in aqueous suspension for 10 min at 60 to 75 C, prior to incubation in glucose, germinated rapidly and completely. Heating in aqueous suspension not only increased the total number of spores germinating, but also the germination rate (11). There appeared to be a critical temperature of activation, with little activation of spores heated for 10 min at temperatures of 50 C or lower. Heating at 80 C reduced activation from maximal levels. Spores were not activated when heated dry (over P_2O_5), but such dry-heated spores could be fully activated subsequently by suspension in water and heating at 65 C for 10 min.

With the failure of elevated temperature to activate dry spores, the question of the role of water in activation arose. Dry lyophilized spores exposed to water vapor at 30 C (a temperature well below that required for heat activation) were activated for subsequent germination in glucose solution (Fig. 1). After 120-min exposure to water vapor at 75 to 90% relative humidity, spores germinated in glucose to almost the same extent as those heated in aqueous suspension at 65 C. Spores

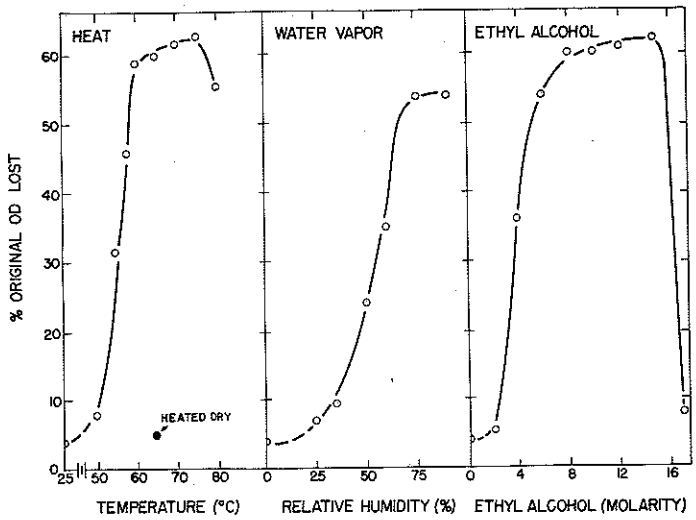

Fig. 1. *Activation of B. megaterium QM B1551 spore germination by heat, water vapor, and aqueous ethyl alcohol. Activation conditions were as follows. Heat: spores were heated in aqueous suspension for 10 min at the indicated temperatures. Water vapor: lyophilized spores were exposed at the indicated relative humidities for 120 min at 30 C, and then suspended in liquid water. Ethyl alcohol: spores were treated with aqueous ethyl alcohol at the indicated concentrations for 5 min at 30 C. Germination was determined after incubation for 120 min at 30 C in 25 mM glucose, buffered with 50 mM potassium phosphate (pH 7.0).*

exposed to water vapor at 50 to 60% relative humidity germinated to approximately the same extent as those heated in aqueous suspension at 55 C for 10 min. The germination rate of water vapor-activated spores (75 or 90% relative humidity) was lower than that of spores activated by heating at 65 C for 10 min (11). However, maximal germination, with germination rate close to that obtained by heat activation, was attained by exposing spores at 60% relative humidity at 30 C for 168 hr (data not shown). After prolonged exposure at higher relative humidities, spores germinated spontaneously, that is, without added substrate.

B. megaterium spores were also activated by incubation in aqueous ethyl alcohol at 30 C for 5 min (Fig. 1). There was little or no activation at concentrations of ethyl alcohol below 2 M, or with absolute alcohol (17.2 M). Spores which had been exposed to 8 to 15 M ethyl alcohol germinated completely in glucose, although their rate of germination was lower than that of maximally heat-activated spores (11).

Germination on L-alanine or KI required much higher levels of heat or water vapor activation than were necessary for glucose-induced germination (11).

The presence of water appeared to be critical to the three activation treatments—heating, ethyl alcohol, and, of course, water vapor. Heating would be expected to change the molecular structure of liquid water so that it became more "vapor-like," either by disruption of hydrogen bonds with consequent decrease in the size of water polymers and increase in the water monomer fraction, or by decrease in the strength of hydrogen bonds (11). We have postulated that the absorption of water by the spore and hydration of a specific spore site which initiates activation (11) is more readily accomplished by water whose structure has been altered by heating so as to resemble gaseous water. Ethyl alcohol might facilitate the entrance of water to specific spore sites by changing water structure or by lowering its surface tension. Since certain anions have been shown to alter water structure, they might function like heating in the activation process (11). The aging phenomenon (11) could be explained on the basis that, on extended storage, "vapor-like" water, present as a small fraction in liquid water, could enter into and hydrate a spore site.

We envision the site of activation as an enzyme or enzyme system, dehydrated and inactive in the dormant spore, the activity of which is required for germination. Activation may involve a reversible denaturation of proteins resulting in an "unblocking" of an enzyme system (12). Such denaturation would occur more readily in a hydrated than in a dehydrated system and perhaps more readily at elevated than at ambient temperatures. Alternatively, an enzyme which has been hydrated by one of the activation treatments may be dissociated (at a temperature-dependent rate) from an inactive complex, perhaps an inactive Ca-DPA-enzyme complex (17), or from a binding site (7), the active enzyme then being able to use added or intrasporal substrates for reactions leading to germination.

If the crucial event in activation is hydration of a specific spore site, removal of water might reverse activation. Attempts to deactivate water vapor-activated spores by desorption under vacuum were unsuccessful. Although more than 99% of the water sorbed during activation (90% relative humidity, 30 C, 30 min) was removed by evacuation for 24 hr, there was no loss of activation for glucose-induced germination. It was possible that the water involved in activation was too firmly bound to be easily removed by vacuum, or was too small to detect. These data suggest that

the site whose hydration might be necessary for spore activation could occupy only a small fraction of the spore volume.

Similarly, other relatively mild dehydration treatments (40 hr at 66 C, or 40 hr over P_2O_5 at 30 C) did not reverse activation by water vapor, but more rigorous dehydration methods [100% ethyl alcohol at 66 C for 18 hr (8) or over P_2O_5 at 66 C for 40 hr (11)] did partially reverse activation by ethyl alcohol, heat, and water vapor. The P_2O_5-deactivated spores (Fig. 2), for example, could be reactivated by reapplication of any of these activation treatments.

It is noteworthy that deactivated spores, treated with 2 mM calcium acetate for 30 min at 30 C, then washed, also regained the ability to germinate in glucose (Fig. 2). Furthermore, activated spores were deactivated by treatment with acid according to the method described by Lee and Ordal (14). These observations prompted us to investigate heat activation and germination of spores converted to various ionic forms.

Activation of ionic forms of B. megaterium. Alteration of spore composition by such methods as modification of the sporulation medium (4, 9, 15), addition of ions to spores undergoing endotrophic sporulation (5), or base exchange techniques (1, 19) results in alteration of spore germination requirements (including the necessity for activation). Changing the spore's chemical content may also differentially affect its germination pattern on different germinants (15). We shall now consider the germi-

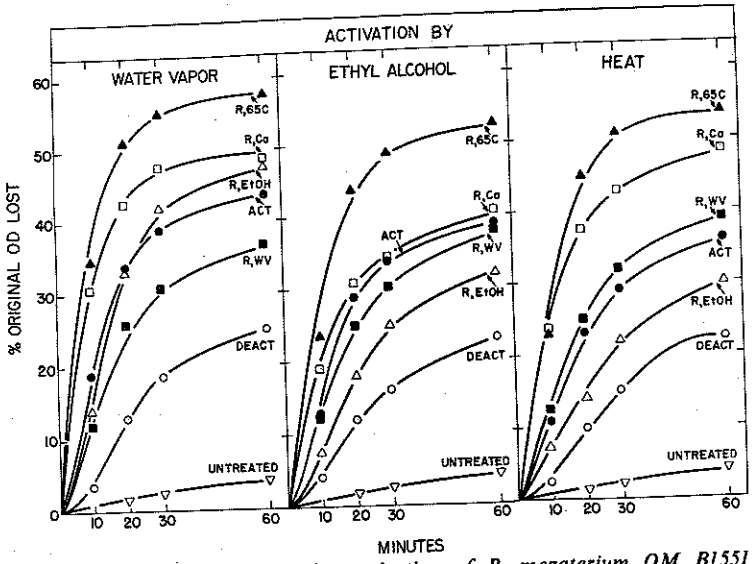

Fig. 2. *Activation, deactivation, and reactivation of B. megaterium QM B1551 spores. Activation (ACT) conditions were as follows. Water vapor: 90% relative humidity, 30 min, 30 C. Ethyl alcohol: 5 M, 5 min, 30 C. Heat: 58 C, 10 min. Deactivation (DEACT) was by 40-hr treatment over P_2O_5 at 66 C. Reactivation (R) was by heating at 65 C, 10 min; by water vapor (WV) or by ethyl alcohol (EtOH), same conditions as for activation; or by Ca^{++}, suspended in 2 mM calcium acetate, 30 C, 30 min; then washed three times. Germination conditions were as in Fig. 1.*

nation on glucose, KI, and L-alanine of ionically altered *B. megaterium* QM B1551 spores, particularly as affected by the application of heat.

The germination of spores of *B. megaterium* QM B1551, adjusted to *p*H 6.0, 4.0, and 2.5, and presumably containing different levels of cations, was investigated. Unheated N-spores germinated to a greater extent with glucose (Fig. 3) than did the pooled spores (Fig. 1), possibly owing to relyophilization or to activation during storage (11). Unheated H-spores showed less glucose-induced germination than N-spores, the extent of the reduction depending on the *p*H titration level. N- and H-spores did not germinate on KI to any extent unless heated. The response of H-spores for L-alanine-induced germination was entirely different from that for glucose-induced germination (Fig. 3). Only 10% of the unheated N-spores germinated on L-alanine (5% OD loss). Germination was increased to 18% by stripping the spores to *p*H 4.0, and to almost 50% by stripping them to *p*H 2.5.

Heat activation was necessary for maximal germination on all substrates. H-spores, however, were more sensitive than N-spores to heat inactivation of germination, the degree of sensitivity depending upon the *p*H to which they had been titrated (Fig. 3). N- and H-6 spores germinated maximally on glucose if they were heat-activated between 60 and 75 C, but germination of the H-6 spores was reduced slightly more than that of N-spores if they were heated at 80 or 85 C. H-4 spores, activated at 60 or 65 C, germinated slightly less than N-spores, and their germina-

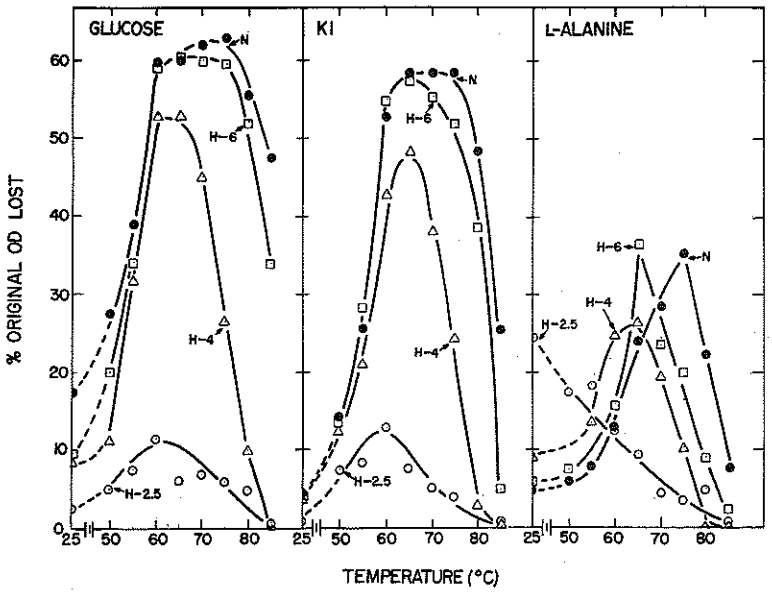

Fig. 3. *Germination of N- and H- spores of B. megaterium QM B1551, heated at various temperatures. N- spores and H- spores, titrated to pH 6, 4, or 2.5, were heated for 10 min. Germination was then determined after incubation for 240 min at 30 C in glucose (25 mM), KI (5 mM), or L-alanine (100 mM). In this, and in succeeding figures, the dashed lines are extrapolated values.*

tion was markedly reduced when heated at 70 C or higher. H-2.5 spores germinated only minimally on glucose. Response of heated H-spores to KI-induced germination was similar to that for glucose-induced germination. N-spores attained their maximal germination on L-alanine when heat-activated at 75 C; H-6 spores, when heated at 65 C; H-4 spores, when heated at 60 C (but this was higher than the germination of N-spores, heated at the same temperature); and H-2.5 spores, when unheated. H-2.5 spores were extremely sensitive to heat inactivation of germination, temperatures as low at 50 C drastically reducing their germination from the high level attained by the unheated spores.

The conversion of spores to the H form had several effects: (i) ability of unheated spores to germinate was decreased on glucose, but increased on L-alanine; and (ii) spore sensitivity to heat inactivation of germination was increased, temperatures of inactivation being dependent on the degree of stripping and on the germination substrate.

If these effects were due to the removal of ions from spores, reloading with ions should reverse these changes (1, 19). The effects on ability to germinate of loading spores with various ions are shown in Fig. 4. Loading with Ca^{++} (and to a lesser degree with K^+ or Na^+) enhanced the glucose-induced germination of unheated or submaximally activated N- or H-4 spores. Loading of H-4 spores with Sr^{++} or

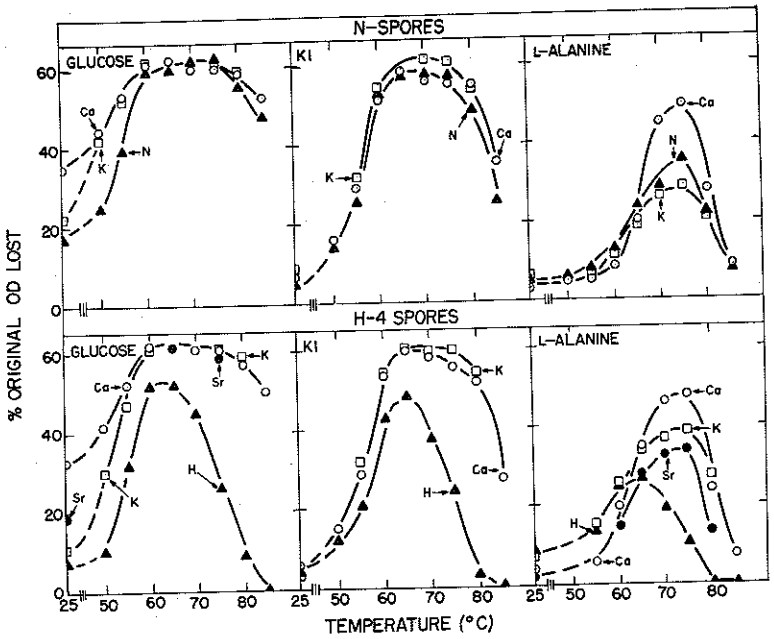

Fig. 4. *Germination of cation-loaded N- and H-4 spores of B. megaterium QM B1551, heated at various temperatures. N- and H-4 spores were loaded by titrating them to pH 9.0 with the hydroxides of the indicated cations. Data for Na-loaded similar to those for K-loaded spores; data for Ba-loaded similar to those for Sr-loaded spores. Heating and germination conditions as in Fig. 3.*

Ba^{++} had less effect than Ca^{++} loading, but slightly more than K^+ or Na^+ loading. Cation loading did not affect the KI-induced germination of unheated N- or H-4 spores; for this reason, germination on this substrate was not further investigated. For L-alanine-induced germination, loading with Ca^{++}, Sr^{++}, or Ba^{++}, but not with K^+ or Na^+, reduced the germination of N- or H-4 spores, either unheated or heated at low temperatures. Although cation loading had different effects on germination of unheated spores or spores heated at low temperature, depending on the substrate, loading with any of the tested ions restored the germinability of H-4 spores heated at higher temperatures, regardless of substrate. However, Ca^{++} was somewhat more effective than the other ions in restoring the germinability of heated H-4 spores on L-alanine.

We had previously observed (15) that spores which were prepared in media supplemented with Ca^{++} were stimulated for glucose-induced germination, but had reduced germination on L-alanine, and that heat activation requirements also depended on the sporulation medium. In the present experiments, we found that Ca^{++} loading by cation-exchange techniques also increased glucose-induced germination, but decreased L-alanine-induced germination. But Ca^{++} appeared to have a dual role, in that, regardless of the germinant, it protected spores against heat inactivation, possibly by protecting proteins which required heating for maximal enzymatic activity. This duality was, perhaps, more clearly seen with spores titrated to *p*H 2.5, where there were greater differences in germination level between N- and H-spores (Fig. 5). The low level of glucose-induced germination of

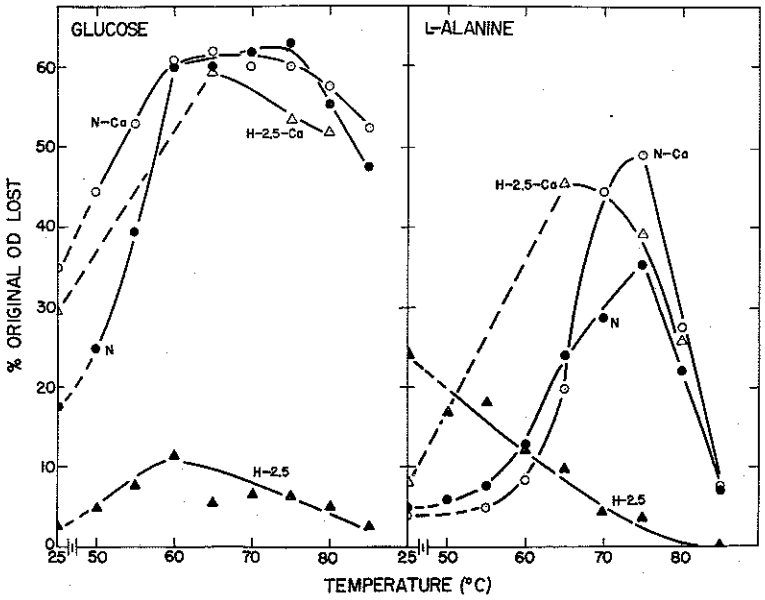

Fig. 5. *Germination of Ca-loaded N- and H-2.5 spores of B. megaterium QM B1551, heated at various temperatures. For loading conditions, see Fig. 4; for heating and germination conditions, see Fig. 3.*

unheated H-2.5 spores was restored by reloading them with Ca^{++}. In contrast, the high level of L-alanine-induced germination of unheated H-2.5 spores was *reduced* by Ca^{++} loading, but the reloaded spores were then less sensitive to heat inactivation.

The dual role of $Ca^{++}$was further elucidated in a series of experiments in which Ca^{++} (as calcium acetate) was added in the germination medium, rather than by loading the spores with Ca^{++} (Fig. 6). MES [2-(*N*-morpholino) ethanesulfonic acid] buffer (6), which replaced phosphate in these experiments, appeared to have a stimulatory effect on L-alanine-induced germination. Glucose-induced germination of unheated H-4 spores was *increased* either by loading them with Ca^{++} or by having Ca^{++} present in the medium in which the H-4 spores were germinating. L-Alanine-induced germination of H-4 spores was *reduced* by either form of Ca^{++} addition. On either glucose or L-alanine, Ca-loaded H-4 spores were protected from heat inactivation of germination, but Ca^{++}, incorporated in the germination medium of H-4 spores which had already been heated, did not appreciably reverse their heat-inactivated ability to germinate.

N-spores, heated at 65 C for 10 min, appeared to have germinated maximally on glucose (Fig. 3-5), but their rate of germination (Table 2) was greatly increased (from 5.1 to 11.2% OD loss/min) by loading with Ca^{++} (but not with K^+ or Na^+).

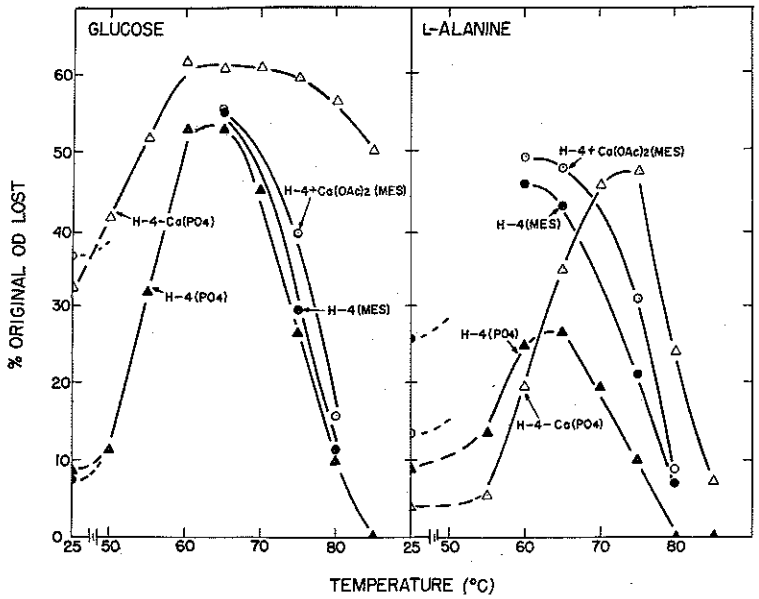

Fig. 6. *Germination of H-4 spores of B. megaterium QM B1551 under various conditions of calcium addition. H-4 spores were either loaded with Ca^{++} by titration to pH 9.0 (see Fig. 4), then heated and germinated as in Fig. 3; or H-4 spores were first heated, then Ca^{++} (2 mM calcium acetate) was added in the germination medium. In the latter case, MES [2-(N-morpholino) ethanesulfonic acid] buffer (6) at pH 7.0 was substituted for phosphate to avoid precipitation of added Ca^{++}.*

Table 2. *Germination rate in glucose of various ionic forms of B. megaterium QM B1551 spores*

Ionic form	Germination rate[a] (% OD loss/min)
N	5.1
N-K	5.3
N-Na	5.25
N-Ca	11.2
H-4	1.09
H-4-K	3.57
H-4-Na	3.3
H-4-Ca	13.1
H-4-Sr	7.4
H-4-Ba	5.8

[a]Germination rate calculated as highest percentage loss of OD per minute after addition of glucose (25 mM) to spores heated at 65 C for 10 min. OD readings were taken at 2-min intervals until the rate leveled off.

Although H-4 spores, heated at 65 C, reached near maximal total germination in glucose in 240 min (Fig. 3 and 4), they germinated very slowly (1.09% OD loss/min). Loading of H-4 spores with Ca^{++} (and to a lesser degree with Sr^{++} or with Ba^{++}) increased their germination rate to a level higher than that of N-spores. Loading with K^+ or Na^+ also increased the germination rate of the H-4 spores, but not to the level of the N-spores. H-4 spores preincubated for 3 hr in potassium phosphate buffer at *p*H 7.0 germinated (upon the addition of glucose) at the same rate as if they had been loaded with K^+ at *p*H 9.0. The near maximal total germination that occurred with extended incubation (240 min) of H-4 spores in glucuse, buffered with potassium phosphate at *p*H 7.0, was probably due to uptake of K^+ by the spores (even at this *p*H level), resulting in an increased germination rate.

Spores produced on media supplemented with Ca^{++} are more heat-resistant than spores grown with only minimal quantities of this metal (15, 21). It has been reported that heat resistance of spores treated with thioglycolate at low *p*H can be restored by incubating such spores in calcium acetate (20), and that exchangeable Ca^{++} is concerned in spore heat resistance (1). In our experience, H-spores were less heat-resistant (84 C, 5 min) than, and Ca-loaded N-spores were more heat-resistant than N-spores (Fig. 7), but the differences were not as great as those reported by Alderton and Snell (1). However, in agreement with these authors (1), we found that loading with Ca^{++} restored the heat resistance of H-spores (Fig. 7) to at least as high a level as that of N-spores.

Rode and Foster (19) found that N- or H- spores of *B. megaterium* strain Texas were unable to germinate in alanine-inosine in the absence of a strong electrolyte. The necessity for an electrolyte was relieved if the spores were loaded with Ca^{++}.

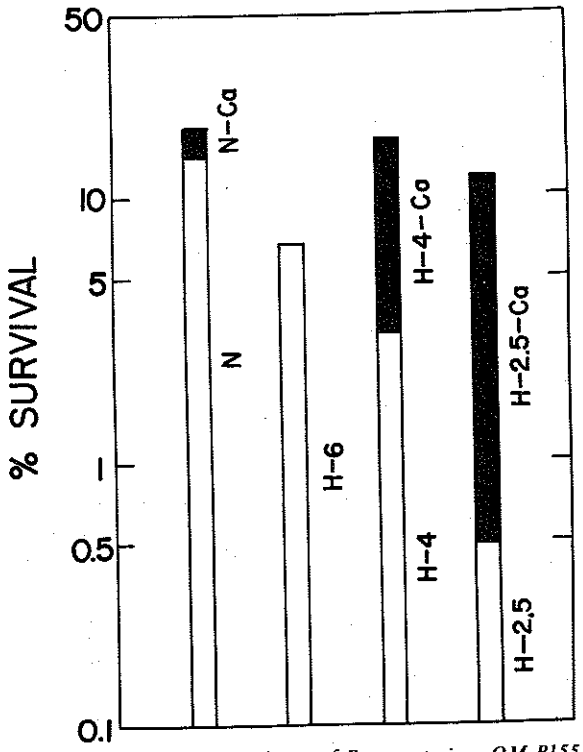

Fig. 7. *Heat resistance of various ionic forms of B. megaterium QM B1551 spores. Aqueous spore suspensions were heated at 84 C for 5 min. Appropriate dilutions of heated spores were plated on Nutrient Agar plus 0.1% yeast extract and incubated for 24 hr at 30 C. Open bars show percentage survival of N- or H-spores. Solid bars show percentage survival of similar spores, loaded with* Ca^{++} *by titration to pH 9.0.*

B. megaterium QM B1551 spores were unable to germinate in L-alanine in the absence of K^+ in the germination medium (*our unpublished data*), but loading with Ca^{++} did not relieve the necessity for K^+. An electrolyte requirement does not appear to form the general basis for the effects of stripping and loading of spores.

Activation, as effected by what we consider to be hydration treatments (heat, aqueous ethyl alcohol, and water vapor) with consequent activity of germination enzymes (7, 23), differs fundamentally from the promotion of germination by the addition of ions. The former, we believe, represents a general concept of activation; the latter represents a specific requirement for germination on a particular substrate. Indeed, an ion which is stimulatory for germination on one substrate (glucose) may inhibit germination on another (L-alanine), but a sufficiently hydrated spore is activated for germination regardless of the specific germinant.

This is not to say, however, that activation by a hydration treatment is unrelated to ionic promotion or diminution of germination. For example, glucose-induced germination of spores which were activated by the hydration treatments could be

decreased either by acid treatment or by dehydration over P_2O_5. The relationship between the increase in germinability as a result of addition of water or ions, and its decrease as a result of removal of water or ions, might well be reexamined in the light of present knowledge concerning mutual interactions in the hydration of colloids and ions.

We previously postulated that the mechanism for germination of *B. megaterium* QM B1551 spores on glucose differs fundamentally from that for germination on L-alanine (10). Rode (18) reported that *B. megaterium* QM B1551 spores, of the glucose-nitrate (GN) germination type, required more acid to titrate them to *p*H 4.0 than did *B. megaterium* spores of the L-alanine-inosine (AL) germination type. This suggested the presence of a greater number of binding sites for H^+ and the presence of more exchangeable cations (e.g., Ca^{++}) on GN spores than on AL spores. Such exchangeable cations on GN spores may promote spore germination on glucose and inhibit their germination on L-alanine. By decreasing the cation content of *B. megaterium* QM B1551 spores, we have been able to manipulate them to have reduced germination on glucose, and increased germination on L-alanine. It is possible that the germination pattern of the AL type of *B. megaterium* might also be alterable by ionic manipulation.

The germination pattern in response to heating represented a balance between activation and inactivation. Exchangeable spore cations had a strong influence on this balance, and, therefore, in effect, on the optimal temperature for heat activation. We believe that the results of other investigators relating to the germination of ionic forms of bacterial spores on a single substrate, or under a single condition of heat activation, might well be reevaluated on the basis of the experiments reported here.

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